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The role of particle-mediated DNA vaccines in biodefense preparedness

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Abstract

Particle-mediated epidermal delivery (PMED) of DNA vaccines is based on the acceleration of DNA-coated gold directly into the cytoplasm and nuclei of living cells of the epidermis, facilitating DNA delivery and gene expression. Professional antigen-presenting cells and keratinocytes in the skin are both targeted, resulting in antigen presentation via direct transfection and cross-priming mechanisms. Only a small number of cells need to be transfected to elicit humoral, cellular and memory responses, requiring only a low DNA dose. In recent years, data have accumulated on the utility of PMED for delivery of DNA vaccines against a number of viral pathogens, including filoviruses, flaviviruses, poxviruses, togaviruses and bunyaviruses. PMED DNA immunization of rodents and nonhuman primates results in the generation of neutralizing antibody, cellular immunity, and protective efficacy against a broad range of viruses of public health concern.

Keywords: DNA vaccine; Gene gun; Epidermis; Biodefense; Ebola; Marburg; Dengue; Smallpox; Tick-borne encephalitis; Venezuelan equine encephalitis; Hantavirus

Contents

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14. ABSTRACT

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15. SUBJECT TERMS

Vaccination, DNA vaccine, epidermal delivery, viral agents, laboratory animals, nonhuman primates,rodents

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1. Introduction

Recently, there has been a resurgence of interest in developing vaccines to protect civilian and military populations against potential biological warfare agents. This effort has increased the focus of research on causative agents for diseases that may have limited incidence or prevalence. Coordinated by the Centers for Disease Control and Prevention, military and civilian working groups have developed methods for assessing potential biological threat agents [1]. Multiple factors were considered to evaluate the threat posed by specific agents, including public health impact (expected untreated morbidity and mortality), agent availability and capability for mass production, potential for initial dissemination to a large population and continued propagation by person-to-person transmission, public perception of risk, and special public health preparedness requirements such as stockpiling of therapeutics, enhanced surveillance and new diagnostic capabilities. Agents were assigned to categories A, B, or C depending on the severity of the potential threat. Among the potential biological threat agents identified in categories A and B are the viral pathogens associated with smallpox, viral hemorrhagic fevers, and viral encephalitis. Viral emerging threat agents are assigned to category C.

This review focuses on recent developments in particle-mediated DNA vaccines with particular emphasis on vaccines to address current and emerging threats due to category A to C viral agents in the families Filoviridae, Poxviridae, Togaviridae and Bunyaviridae, and on vaccines against viruses of the family Flaviviridae. The combination of potential high morbidity and mortality, lack of effective antiviral treatments, and weaponization potential of these agents has resulted in increased efforts for development of new or improved vaccination strategies. Development of conventional modified-live or killed vaccines for these agents is in many cases not feasible due to significant safety concerns associated with potential reversion to virulence or incomplete virus inactivation. Manufacturing of conventional vaccines from highly pathogenic agents is also complicated by safety concerns associated with potential operator exposure during bulk virus manufacturing, which must be conducted in high-level biocontainment facilities that are costly and difficult to build and operate.

The use of DNA vaccine technology precludes the need for handling of hazardous viral pathogens, as only the DNA encoding protective antigens is incorporated into the vaccine. DNA vaccine technology thus eliminated the need for biocontainment, and the risk of exposure to live viral agents. Several modalities have been employed to deliver DNA vaccines, including intramuscular (i.m.) and intradermal (i.d.) routes using conventional needle and syringe or the needle-free Biojector. This review will focus specifically on delivery of DNA vaccines to the epidermis using particle-mediated epidermal delivery (PMED) of DNA vaccines to address current and emerging threats due to viral agents. PMED has been employed to assess feasibility of DNA vaccine development against a number of pathogens, where neutralizing antibody, cellular immunity, and protective efficacy have been demonstrated in preclinical models.

2. Particle-mediated DNA vaccines

2.1. Basic mechanism of epidermal delivery

Particle-mediated DNA delivery systems (often referred to as "gene guns") have been employed since the mid-1980s for the direct intracellular delivery of DNA into both cultured cells and whole organisms for purposes of transient and stable transgene expression, plant genetic engineering, gene therapy, and DNA vaccine delivery (for early references, see [2–8]). The physical, rather than biological nature of the delivery process, permits gene expression in essentially any cell type from yeast to plants and animals. Whether employing the motive force of an electric discharge or compressed helium, particle-mediated DNA delivery systems are all based on the acceleration of DNA-coated gold or tungsten particles directly into the cytoplasm and nuclei of living cells, facilitating DNA delivery and gene expression. Importantly, use of this technology requires direct access to the target cells in question. Thus, while systemic gene delivery in humans is not feasible with particle delivery systems, the skin is readily accessible and is recognized as an important immunologically inductive site for the targeting of DNA vaccines [9,10]. Therefore, in terms of clinical applications, PMED of DNA vaccines has become the logical focus for this DNA delivery platform because of the ease of penetrating the outer layer of skin, the stratum corneum, and achieving the direct intracellular delivery of DNA vaccines into keratinocytes and epidermal Langerhans cells of the viable epidermis (Fig. 1).

2.2. Particle-mediated epidermal delivery versus intramuscular inoculation of DNA

While the majority of DNA vaccine reports have been based on the i.m. inoculation of naked DNA in the absence of any intracellular delivery system, PMED was employed in the very first DNA vaccine (genetic immunization) report in 1992 [8] and has received widespread attention because it can often achieve stronger immune responses with two to three orders of magnitude less DNA than required in i.m. inoculation studies [11–15]. This marked difference in the efficiency of immune response induction per unit of DNA administered is almost certainly due to the use of a physical, intracellular delivery system that can propel the DNA vaccine directly into the cell cytoplasm or nucleus. While i.m. DNA vaccine performance can decline when moving from mice to larger animals, PMED DNA vaccines enable the induction of significant responses in large animals such as pigs and monkeys [10,11,16–19]. Moreover, in humans where DNA doses up to 5 mg administered via i.m. inoculation result in only modest cellular responses and essentially no antibody responses [20–28], PMED DNA vaccines have achieved greater success in inducing both humoral and cellular responses using less than 10 μg DNA [29,30].

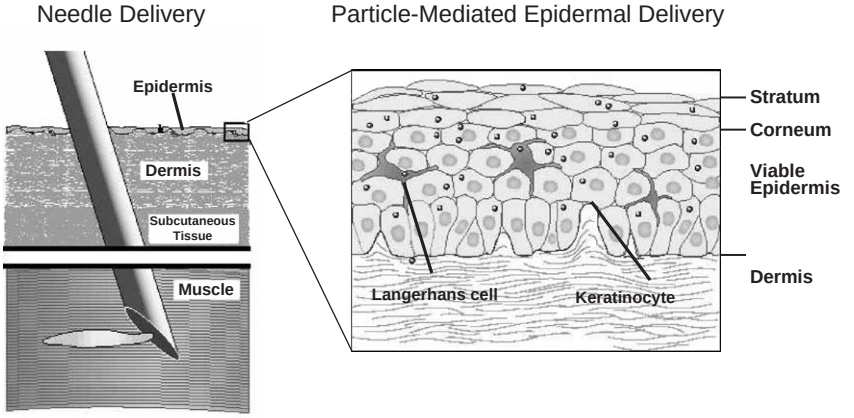

Fig. 1. Mechanism of particle-mediated epidermal delivery of DNA vaccines. DNA on 1- to 3-μm gold particles penetrates through the skin and transfects both Langerhans cells and keratinocytes of the viable epidermis.

The ability of PMED DNA vaccines to elicit immune responses with such small amounts of DNA allows multiple plasmids/genes to be simultaneously delivered for the induction of immunity to multiple antigens [31]. These early data suggest the potential application of PMED DNA vaccine technology to develop combination vaccines. In addition, the PMED platform has been used for identifying protective antigens of complex pathogens in experiments in which multiple antigens derived from expression libraries are simultaneously administered to animals [32–37].

2.3. Mechanisms of antigen presentation

Immune responses to PMED DNA vaccines are initiated by transfection of resident antigen-presenting and non-antigen-presenting cells in the viable epidermis (Fig. 2). Insofar as the efficacy of PMED DNA vaccines is influenced by the use of a direct intracellular delivery system, the epidermis as a target site also contributes to DNA vaccine efficacy because of its resident population of epidermal dendritic cells, the Langerhans cells (LC), and their ability to process foreign antigens and present them in the draining lymph node (DLN). Dendritic cells containing gold particles and expressing the gene of interest have been identified in the draining lymph nodes of animals following PMED DNA vaccination of the skin [38–43]. Moreover, in adoptive transfer experiments, the skin-derived migratory antigen-presenting cells that contribute to the induction of cellular responses were shown to be of bone marrow origin [44], consistent with their identification as epidermal LCs.

In skin ablation and skin grafting experiments following PMED DNA vaccination, it was shown that cells migrating out of the skin delivery site in the first few hours following delivery are responsible for inducing primary cellular responses and immunological memory. In contrast, the non-migratory cells (keratinocytes) that produce antigen at the skin target site contribute predominantly to the magnitude of the antibody response [45,46]. There is strong evidence for the role of antigen expression in both types of cells in that studies investigating the exclusive expression in either antigen-presenting or non-antigen-presenting cells demonstrate responses that are generally reduced as compared to animals receiving DNA vaccines that are not tissue specific [42,47].

In an initial attempt at quantifying the number of successfully transfected dendritic cells that migrate from the skin into DLNs, multiple non-overlapping vaccine doses were administered to mouse abdominal skin. Only 50–100 directly transfected DC could be identified in DLNs 24 h following delivery, however, many times more apparently non-transfected DC migrated into the DLN merely as a function of the stimulatory nature of gold delivery to the skin [40]. This stimulatory aspect of PMED vaccination was independent of DNA attachment to gold. In the same study, immune responses induced following PMED DNA vaccination were shown to be due to antigen production in the directly transfected DCs versus a cross-priming mechanism in which extracellular antigen produced by cells other than the DCs (such as keratinocytes) is picked up and processed by the DCs [40]. This was inferred from the observation that antigen-specific antigen-presenting cells in the DLN could be depleted by antibodies to intact antigen on their cell surface showing that antigen was produced de novo within these cells.

Further evidence that directly transfected DCs contribute to the induction of humoral and cellular responses was derived from studies in which transfer of at least 500–1000 in vitro transfected skin DC induced humoral and cellular responses that were as strong as those elicited via direct PMED DNA vaccination of mice [48]. The requirement for the injection of 500–1000 in vitro transfected DC to elicit the full response implies that the 50–100 observed transgenic DC found in DLNs following PMED DNA vaccination of the skin [40] likely does not represent the full extent of antigen-presenting activity.

Directly transfected DC were also shown to be important in another adoptive transfer experiment involving a drug-inducible promoter that could be turned on at will in recipient mice. In this study, migratory skin cells isolated from donor mice immunized with an inducible promoter plasmid were collected and transferred into recipient mice, after which gene expression was induced de novo in the DCs in recipient animals by treatment with the drug RU486. The newly induced antigen expression in the transfected donor DCs in recipient animals stimulated strong cellular responses, but humoral

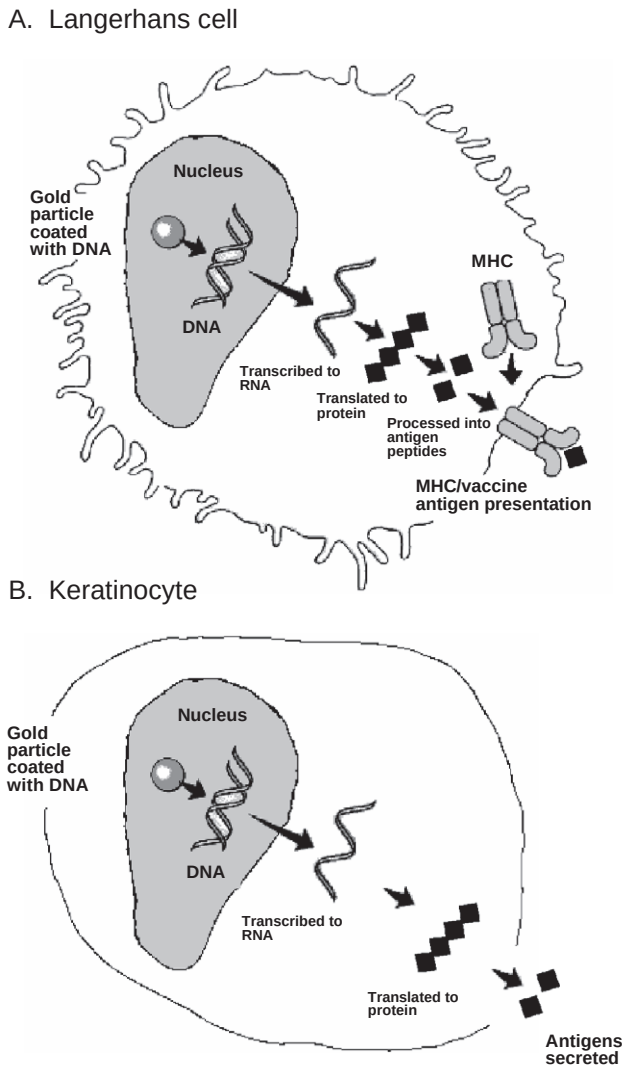

Fig. 2. Antigen presentation in response to PMED DNA vaccines. (A) Gold particles coated with DNA transfect antigen-presenting cells of the epidermis (Langerhans cells). Following expression of the transfected DNA, endogenously synthesized protein antigen is processed in the cytosol into peptides, which are transported to the endoplasmic reticulum and loaded onto MHC class I molecules. This generates transport-competent complexes that then move to the surface of the antigen-presenting cell. (B) Transfection of non-antigen-presenting keratinocytes leads to expression of the plasmid-encoded gene(s) and secretion of the resultant protein antigen. This exogenous antigen is subsequently endocytosed by the Langerhans cells, and processed via the MHC class II pathway (reviewed in [202]) .

responses that were weaker than those observed in directly DNA-vaccinated animals [42]. Because antigen expression was not induced until the migratory skin cells from donor mice were transferred into recipients, a cross-priming mechanism of antigen transfer from non-antigen-presenting cells such as keratinocytes could not have come into play in this case.

To more accurately address the issue of the number of directly transfected DC that migrate from the skin to the DLN, the Cre-Indicator transgenic mouse strain ROSA26R was used to enable the genetic tagging of DC in the skin of these mice following administration of a plasmid encoding the Cre recombinase [49]. The delivery of the Cre plasmid into DC in this transgenic line results in a directed recombination at the trans-

gene locus activating β-gal expression. Very little Cre recombinase expression is required to activate β-gal, making this a much more sensitive technique to identify the numbers of successfully transfected DC. Using this technique, the numbers of tagged DC migrating from the skin to the DLN were approximately 100-fold higher than observed previously [40]. This discrepancy was likely due to a combination of greater sensitivity of β-gal expression using flow cytometry and the ability to detect DC containing only very low amounts of the administered CMV-Cre plasmid since the Cre recombinase need not be expressed at a high level to induce the permanent recombination event that results in high-level β-gal expression. Thus, many more antigen-expressing DC likely migrate to the DLN than originally thought.

While there is a wealth of evidence demonstrating the role of direct APC transfection following PMED DNA vaccination, there is still evidence that cross-priming can contribute to the induced responses. Cho et al. [47] showed, by using non-APC and APC specific promoters and adoptive transfer studies that significant humoral and cellular responses could be elicited by cross-priming mechanism. These studies are consistent with others showing that in all likelihood, both cross-priming and direct APC transfection contribute to the complete spectrum of humoral, cellular and memory responses [45].

2.4. Polarization of immune responses to DNA vaccines

Current dogma in the field holds that PMED DNA vaccines elicit Th2 responses while i.m. inoculation of naked DNA elicits Th1 responses. This initial observation stems from several studies in mice in which such a pattern was observed with certain antigens [11,50–52] with some variation in the nature of the responses attributed to the identity or form (secreted, membrane bound, cytoplasmic) of the antigen [52,53]. Further investigation has revealed a more complicated situation, in that responses to some antigens in mice are clearly more balanced between Th1 and Th2 [9,10,53,54]. Strong CTL responses and Th1 cytokine production were demonstrated in mice following PMED DNA vaccination [14,38,53,55] and in one specific case, the strongest CD8 T cell responses and the lowest DNA dose requirement were observed in PMED DNA-vaccinated mice, as compared to i.m. vaccinated mice [56].

The tendency toward Th2-like responses in mice for some antigens may be a characteristic of mice since PMED DNA vaccines, when directly tested in nonhuman primates, clearly induce responses that are more Th1-like in character and provide challenge protection [17,57]. Also, in prime boost regimens in nonhuman primates (see below) priming via PMED results in robust Th1-like CD4+ and CD8+ responses upon recombinant viral vector boosting [57–60]. Finally, Th1 responses characterized by IFN-γ production and CD8+ effector T cell responses were demonstrated in humans in a hepatitis B surface antigen PMED DNA vaccine trial [29].

Polarization of the immune response toward Th1 or Th2 can therefore be influenced by several factors, including the nature of the antigen, addition of immunostimulators, dosing regimen [54,61–63], and target species. While the types of responses elicited via PMED DNA vaccination have been the focus of significant attention, the biological significance of these responses in rodents, nonhuman primates, and other species has been amply demonstrated in numerous challenge trials. Examples of PMED-mediated protective immunity have been observed with viral, bacterial and parasite pathogens (Table 1).

For the category A–C viral pathogens, most vaccine development efforts have been focused on strategies to elicit high-titer, long-lasting neutralizing antibody responses. Limitations to model systems and lack of human data have hampered investigation of the role of cellular responses in protective efficacy. However, some studies have demonstrated a direct correlation between cellular responses and protection, or inferred the importance of cellular responses due to evidence of protection in the absence of neutralizing antibody. For example, emerging data suggest that it is difficult to elicit high-titer neutralizing antibodies against Ebola virus, however, preclinical studies have shown a correlation between cellular immune responses (in addition to neutralizing antibody responses) and protection [64–66]. In addition, a dengue DNA vaccine was shown to elicit cytotoxic T lymphocyte responses in nonhuman primates. After challenge, protection was observed in both monkeys with and without detectable neutralizing antibody responses [67]. When mice are vaccinated with a

Table 1
Efficacy of PMED DNA vaccines in preclinical models

Agent	References
Virus	
Flaviviruses	Schmaljohn et al., 1997; Colombage et al., 1998; Schmaljohn et al.; 1999, Pan et al., 2001; Putnak et al., 2003 [67,137,140,153,154]
Filoviruses	Vanderzanden et al., 1998; Hevey et al., 2002; Mellquist-Riemenschneider et al., 2003; Riemenschneider, 2003 [31,65,141,129]
Poxviruses	Hooper et al., 2000; Hooper et al., 2003 [164,14]
Hantaviruses	Hooper et al., 1999; Kamrud et al., 1999; Hooper et al., 2001; Custer et al., 2003 [176–178,180]
Venezuelan equine encephalitis virus	Riemenschneider et al., 2003 [31]
Papillomaviruses	Stanley et al., 2001; Sundarum et al., 1997; Moore et al., 2002; Kim et al., 2003; Kim et al., 2004 [95,97,184–187]
Rabies virus	Lodmell et al., 2001; Lodmell et al., 2002 [188,189]
Foot and mouth disease virus	Benvenisti et al., 2001 [16]
Influenza virus	Fynan et al., 1993; Webster et al., 1994; Macklin et al., 1998; Kodihalli et al., 1997 [12,18,190,191]
Immunodeficiency viruses	Fuller et al., 1997; Fuller et al., 2002; Kent et al., 2002 [17,57,106]
Herpesviruses	Kondo et al., 2004 [192]
Bacteria	
Bacillus anthracis	Price et al., 2001; Riemenschneider et al., 2003 [31,193]
Pseudomonas aeruginosa	Price et al., 2001; Price et al., 2003; Staczek et al., 2003 [194–196]
Listeria monocytogenes	Yoshida et al., 2001 [197]
Borrelia burgdorferi	Scheiblhofer et al., 2003 [198]
Mycobacteria	
Mycoplasma pulmonis	Lai et al., 1995 [199]
Parasite	
Plasmodium falciparum	Sakai et al., 2003; Rainczuk et al, 2003; Weiss et al., 2000; Leitner et al., 1997 [54,84,200,201]

conventional Venezuelan equine encephalitis vaccine, protection is associated with high levels of neutralizing antibody, while mice vaccinated with PMED DNA vaccine can withstand challenge infection in the presence of low neutralizing antibody responses, suggesting that either non-neutralizing antibody or cellular immune responses were mediating protection [31]. Even where correlation between cellular immune responses and protection has not been demonstrated, in most cases, the goal is to develop vaccines capable of eliciting both neutralizing antibody and cellular responses, in order to improve potential protective efficacy and persistence of memory cells.

2.5. Relevance of CpG to PMED DNA vaccines

It has been known for some time that bacterial DNA is recognizably different from mammalian DNA due to the markedly higher concentration of unmethylated CpG dinucleotide motifs [68,69]. These elements are recognized by the innate immune system via toll-like receptor 9 (TLR9) and lead to activation of macrophages, dendritic cells, and B lymphocytes following endocytic uptake of CpG-containing DNA. The presence of CpGs in the bacterial plasmid backbones of essentially all DNA vaccine plasmids is generally believed to contribute to the immunogenicity of DNA vaccines by the simultaneous activation of both the innate and adaptive arms of the immune system [70–73]. However, there is also conflicting evidence that CpG motifs are not required for the induction of Th1 immune responses in mice receiving naked DNA vaccine by i.m. inoculation [74].

Immunostimulatory effects of CpG motifs have not been reported for DNA vaccines delivered by the PMED route. Based on the mechanism of CpG activation and the requirement for endocytic uptake, one would not predict that direct cytoplasmic or nuclear delivery of CpG-containing plasmids would lead to TLR9 activation following PMED DNA vaccination. In one study, it was shown that intradermal (i.d.) needle inoculation of CpG-containing oligonucleotides near the site of DNA/gold delivery resulted in a significant immunostimulatory effect [75], and a marginal immunostimulatory effect due to gold particle-mediated CpG delivery was reported by Zhou et al. [76], however, there is no clear evidence for the importance of CpG motifs in PMED DNA vaccines. It has been proposed that the low DNA dose employed in the PMED route provides an insufficient amount of immunostimulatory CpG motifs (see below) to result in Th1 responses. However, Weiss et al. demonstrated that large PMED DNA doses do not induce responses with a greater

Th1 character [77]. Moreover, at least in mice, the act of delivering gold itself seems responsible for the apparent Th2 tendency observed for certain antigens in this species [77].

2.6. Clinical results with PMED DNA vaccines

The first demonstration of the induction of humoral and cellular immune responses in humans via the PMED DNA vaccine route came from a Phase 1 trial of a hepatitis B surface antigen (HBsAg) DNA vaccine [29]. In this study, 12 of 12 HBsAg naïve subjects developed protective levels of HBsAg-specific antibody titers and all exhibited both CD4+ and CD8+ IFN-γ ELISPOT responses following a 3-dose regimen of either 1, 2 or 4 μg DNA per dose. Although the antibody titers induced in this trial were not as strong as those elicited by the conventional vaccines, the 100% seroconversion is a notable contrast to results of other DNA vaccine studies in which i.m. injection of as much as 5 mg of DNA resulted in low or no antibody responses [20–28]. The immunogenicity of the PMED DNA vaccine with up to 1000-fold less DNA per immunization is likely due to the efficiency of the intracellular delivery system and the ability to target the highly active immunologically inductive epidermal tissue.

Conventional recombinant hepatitis B vaccines generally induce protective level antibody titers in the majority of individuals, but varying percentages of subjects are nonresponsive following the receipt of one or more 3-dose regimens and remain unprotected [78–81]. Because animal studies have demonstrated that HBV DNA vaccines can induce HBsAg-specific responses in mouse strains that are normally non-responsive to HBV vaccines [82], a second Phase 1 study was conducted to evaluate the ability of the HBV PMED DNA vaccine to seroconvert HBV nonresponders. Five of the 16 subjects were sAg-nonresponders who had previously failed to seroconvert to a standard 3-dose HBsAg vaccine regimen. A further 6 of the 16 subjects were profound non-responders who failed to reach protective level titers after 6 to 9 conventional vaccine doses. Finally, a third cohort of 5 previously vaccinated subjects whose titers had waned was also included. A single PMED DNA vaccine dose was all that was required to achieve protective level titers in 4/5 sAg-nonresponders and in 5/5 subjects with waned titers. The immune response profile against the PMED vaccine was markedly different than the response to the conventional vaccine. A prime boost effect due to synergy between the conventional and DNA vaccines cannot be discounted here. In the 6-patient group of profound nonresponders, 2 of 6 subjects developed protective levels titers following 1 or 2 PMED DNA vaccine doses. Although it was disappointing that only one-third of the profound nonresponders became protected, the PMED DNA vaccine approach does appear to offer an alternative vaccine strategy that could protect a greater percentage of subjects than is currently protected with the conventional vaccine.

The PMED device was also employed in a clinical trial investigating a malaria DNA vaccine prime boost regimen in conjunction with a recombinant viral vector [83]. In this study, the *P. falciparum* TRAP gene was fused to a synthetic sequence encoding a string of additional malaria B and T cell epitopes and inserted into a standard CMV promoter-based DNA expression plasmid. The recombinant viral vector booster used in this trial, modified vaccinia virus Ankara (MVA), contained the same hybrid TRAP-epitope fusion gene contained in the DNA vaccine. Modest T cell ELISPOT responses were seen in both the PMED (4 μg per dose) and i.m. inoculation (500–2000 μg per dose) groups following the administration of up to three priming DNA vaccine doses. Upon boosting with the recombinant MVA vector, an elevation in T cell responses was observed in all subjects. By comparing groups that received the same MVA boosting dose, PMED DNA vaccine priming resulted in post-boost T cell responses that were several-fold higher than those obtained by priming with much larger doses of DNA administered by needle inoculation. These results are consistent with the earlier human trials demonstrating that PMED of a DNA vaccine can elicit immune responses in humans while requiring only microgram levels of DNA.

While the clinical feasibility of PMED DNA vaccination has been established, several important questions remain to be addressed before the practical application of the technology to biodefense vaccines can be determined. While the dose of DNA required for PMED is lower that that required for IM immunization, the capacity of the prototype delivery devices is also low, resulting in some cases in the

requirement for multiple dosings. The processes for large-scale manufacturing of PMED DNA vaccines have not yet been developed. Further work will be required to determine long-term stability of PMED DNA vaccines. Finally, although the principle of multi-use jet injection devices for military immunization campaigns has been established, the specific technologies and procedures for application to PMED DNA vaccines remain to be developed.

2.7. Inclusion of immunostimulatory agents in DNA vaccines

DNA vaccines, independent of the method of delivery, likely will benefit from the development of formulations that result in greater immunostimulation, and hence, more robust humoral and cellular responses to the encoded antigens. One approach employs co-delivery of plasmids encoding immunostimulatory agents such as cytokines or chemokines. Consistent with data demonstrating the role of IL-12 in augmenting Th1 cellular immunity, plasmids encoding IL-12 can enhance cellular responses when co-delivered with a PMED DNA vaccine [61,84,85]. Positive effects with plasmids encoding IFN-γ, GM-CSF and interferon regulatory factors have also been reported [86–88]. However, while cytokine gene co-delivery has shown promise, even more robust immunostimulatory effects with PMED DNA vaccines have been achieved by co-delivery of plasmids encoding cholera toxin (CT) and the *Escherichia coli* heat-labile enterotoxin (LT) [89]. CT and LT (in their native protein form) have been shown for many years to exhibit potent systemic and mucosal adjuvant effects when formulated with various antigens. The use of CT and LT protein as vaccine adjuvants has been complicated by their toxicity and numerous research efforts have focused on engineering mutants of CT and LT that retain adjuvant potential but with reduced toxicity [90]. In contrast, plasmids encoding wild-type CT and LT can be delivered into the skin of mice and pigs without toxicity, allowing for the realization of the full immunostimulatory potential of CT and LT in the DNA vaccine formulation. More recently, these augmented cellular responses were shown to result in enhanced protection in an HSV-2 challenge model in mice (J. Haynes, unpublished information).

Co-delivery of plasmids encoding apoptosis-inducing factors also augments cellular responses and to a lesser extent, humoral responses to both PMED and i.m. DNA vaccines [91–93]. In this strategy, plasmids encoding mutant caspases are co-delivered along with an antigen gene in order to induce apoptosis in antigen-expressing cells with the intention of making these cells more attractive targets for uptake by professional APCs. Mutant caspases with reduced activity are required in order to allow enough antigen to be expressed prior to the induction of apoptosis. Alphavirus replicons delivered via PMED can also induce stronger responses via a related mechanism. Rather than increasing the level of antigen production, these replicons induce the formation of dsRNA in the transfected cell resulting in the induction of apoptosis and innate immunity [94]. Paradoxically, genes encoding anti-apoptotic factors also induce a significant increase in cellular immunity elicited via PMED [95]. Markedly increased CD8+ T cell responses were demonstrated and it is proposed that anti-apoptotic factors inhibit the initiation of apoptosis in DCs that are migrating from the skin to the draining lymph node. The ability of both pro- and anti-apoptotic factors to augment PMED DNA vaccines likely lies in different cellular sites of action of these divergent strategies [93] with pro-apoptotic signals functioning in non-APCs and anti-apoptotic signals functioning in professional APCs.

In addition to the use of supplementary plasmids encoding specific proteins with immunostimulatory potential (e.g., CT, LT, and pro- or anti-apoptotic factors), alternative strategies employing antigen fusion proteins have been used to target model antigens to specific subcellular compartments to enhance antigen processing and presentation. In one example, stronger cellular responses were demonstrated to a fusion protein comprised of the antigen and an endosomal/lysosomal targeting signal [96]. This strategy can be combined with the anti-apoptotic approach to achieve additional augmentation of cellular responses [97]. Antigens have also been fused to heat shock proteins [56,98], bacterial toxin translocation domains [99], calreticulin [100], CTLA4 [101,102], and herpes virus VP22 [103,104] resulting in significantly augmented immune responses.

2.8. Prime boost vaccination strategies

In the quest to improve immune responses to DNA vaccines, the adoption of a combined vaccine regimen employing one or more "priming" immunizations with a DNA vaccine and one or more "booster" immunizations with a second form of antigen is proving effective in augmenting responses in a number of animal models (and humans [83], see above). While the combination of PMED DNA vaccine priming with recombinant subunit protein boosting has resulted in enhancement of humoral responses in immunodeficiency virus models [63,105], the more common approach is to combine DNA vaccines with recombinant viral vectors in a prime/boost regimen. The earliest reports of the DNA vaccine/viral vector prime/boost regimen employed the PMED route and demonstrated improvements in humoral and cellular responses in monkeys [62,106,107]. In addition, several studies employing i.m. or i.d. inoculation of larger DNA doses as the method of priming were more recently published [108–115]. As demonstrated with prime boost regimens employing i.m. or i.d. inoculation of naked DNA as the means of priming, the use of a PMED device to deliver the priming DNA vaccine doses results in improvements in cellular as well as humoral immune responses upon viral vector boosting [59,60,62,116–118]. In the mouse model, cellular immunity was augmented to both HIV [60] and malaria epitopes [118] by poxvirus vector boosting following PMED DNA vaccine priming. In the latter case, these responses were associated with protection against malaria sporozoite challenge [118]. In addition, in the monkey SIV/HIV model, recombinant poxvirus boosting following PMED DNA vaccine priming resulted in augmentation of cellular immune responses [59,116,117] that were as strong as those observed in SIV infected macaques [117]. In a few cases, enhanced responses as a result of a PMED/poxvirus prime boost regimen resulted in varying degrees of protection against recombinant SIV/HIV challenge [57,58,106,116], while others suggested that i.d. inoculation was superior to PMED DNA vaccine priming in providing protection against SHIV challenge [115]. While the mechanisms behind the augmented humoral and cellular responses associated with prime/boost regimens are poorly understood, some of the strongest immunodeficiency virus-specific responses have been induced to date using this approach, and evidence of the biological efficacy of these responses has been demonstrated in nonhuman primates. These approaches are now working their way into the clinic for the real test of their applicability for some of the more recalcitrant human infectious disease targets.

3. DNA vaccines for category A–C viral agents

3.1. Filovirus DNA vaccines

The filoviruses, including Marburg and Ebola viruses, are enveloped, negative-strand RNA viruses, and are among the most lethal known human pathogens. The rapid disease progression allows little opportunity to develop natural immunity following infection. Little information is available about potential mechanisms of protective immunity in recovered patients. Antibody can arise following infection, but it is late in the course of disease and titers are not always high. Due to the rapid course of disease, it is hypothesized that the ability to elicit cellular immune responses will be an important component of a successful vaccine, however, little information on cellular responses in recovered patients is available. Despite the lack of information about protective mechanisms, several promising vaccine approaches have been explored in recent years. These include a prime boost approach with DNA and adenovirus vectors expressing Ebola virus glycoprotein [64], Venezuelan equine encephalitis replicons expressing Marburg virus glycoprotein and nucleoprotein [119], and an adenovirus vector expressing Ebola virus glycoprotein [120].

The Ebola and Marburg viruses encode 7 proteins. [121,122], and the majority of the viral structural proteins have been tested as candidate vaccine antigens in rodents [123]. Most vaccine efforts have focused on the glycoprotein (GP), since it is the only viral surface-associated antigen, and the nucleocapsid protein (NP). Several animal models exist to evaluate filovirus vaccines. Marburg and Ebola viruses are lethal for several species of nonhuman primates. Both viruses have been adapted for replication in guinea pigs, and a mutant Ebola virus lethal for mice has

been selected ([65], reviewed by [123]). Protection in animal models has been demonstrated with vaccines containing either GP or NP [124,125]. For Ebola virus, but not Marburg virus, the GP also is produced in a nonstructural secreted form, sGP, which differs from GP at the carboxy terminus due to the use of an alternative reading frame [126,127]. The Marburg GP and Ebola GP, sGP, and NP antigens have been tested as potential DNA vaccine candidates, both by i.m. injection and PMED delivery.

Protection against Ebola virus was achieved in guinea pigs by i.m. immunization with plasmids encoding GP, sGP, or NP. Vaccines expressing GP provided more complete protection than those expressing NP alone. The DNA vaccines elicited antibodies detected by ELISA [66]. Sterilizing immunity to challenge of cynomolgus macaques has been achieved with a prime boost immunization strategy [64]. This strategy employed a multi-component vaccine expressing Ebola virus GP from four different virus isolates. This cocktail was delivered as three immunizations of 1 mg of each plasmid (4 mg DNA total). The first and second immunizations were delivered i.m, and the third was delivered i.m. using the needle-free Biojector device. This regimen was followed by a fourth immunization with an adenovirus vector expressing Ebola virus GP. Cellular and humoral immune responses to the vaccination regimen were observed, and both contributed to viral clearance. This work was subsequently extended to demonstrate that the adenovirus-vectored vaccine alone could facilitate more rapid onset of protective immunity than the prime boost regime, though antibody responses were lower [120].

DNA vaccines delivered by PMED have been evaluated for efficacy against Marburg and Ebola viruses in rodents. DNA vaccines expressing either Ebola virus GP or NP protect against challenge infection in mice [65]. In this study, neutralizing antibody titers were low, but strong cytotoxic T cell responses were elicited by immunization (Fig. 3). Complete protection in mice from mortality following challenge was elicited using 0.5 μg PMED DNA vaccine priming dose followed by three boosters of 1.5 μg DNA, also via PMED. In subsequent studies, 3 vaccinations of either GP or NP DNA were found to reproducibly protect all mice from Ebola virus challenge (Fig. 4). Increasing the time between vaccination and challenge decreased efficacy, but at least partial protection was demonstrated against challenge at 9 months post-immunization. These results were subsequently confirmed and the dosing regimen accelerated. In three separate mouse studies, robust antibody responses and complete protection were achieved after two vaccinations at a 4-week interval with a 10 μg dose of either the Ebola virus GP or NP PMED DNA vaccine [31].

A PMED DNA vaccine expressing the GP gene of Marburg virus has been evaluated for immunogenicity and efficacy in rodents and nonhuman primates. The GP vaccine delivered by PMED at a concentration of 4 μg DNA was compared to various other vaccination approaches, including whole killed Marburg virus, live-attenuated Marburg virus, baculovirus expressed Marburg GP, alphavirus replicon-expressed GP, and a DNA prime-baculovirus antigen boost [128]. Guinea pigs were immunized with the various vaccines and challenged with Marburg virus. Regimens with protective efficacy included the whole killed virus vaccine and the DNA prime-baculovirus boost. This same strategy, however, was not effective in protecting monkeys from Marburg virus challenge (C. Schmaljohn, unpublished information). Unlike the Marburg DNA prime-baculovirus boost vaccine, guinea pigs vaccinated by PMED with an Ebola virus GP DNA vaccine and boosted with baculovirus-derived GP were not protected from Ebola virus challenge, despite development of antibodies to Ebola virus detected by ELISA. Therefore, the DNA prime-protein boost vaccine regimen that was protective against Marburg virus in guinea pigs was less effective against Ebola virus.

In a separate study, DNA vaccines expressing GP from two different strains of Marburg virus (Musoke or Ravn) were evaluated for efficacy in a guinea pig model [31]. Guinea pigs were immunized three times at 4-week intervals with a 10 μg PMED DNA vaccine. All vaccinated animals developed antibodies to Marburg virus (Fig. 5). Following homologous Marburg virus challenge, all control guinea pigs developed viremia and died, while none of the vaccinated guinea pigs developed viremia, and all were healthy through the observation period. The DNA vaccine for Marburg virus (Musoke) was also evaluated in cynomolgus macaques [129]. In two individual studies, three monkeys were vaccinated three times

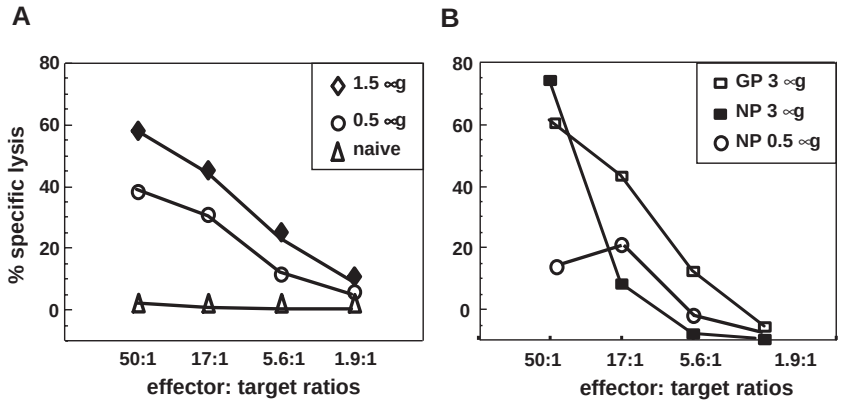

Fig. 3. CTL responses of mice vaccinated with Ebola virus GP DNA. Mice were vaccinated at 4-week intervals by particle bombardment of the epidermis at 4-week intervals with approximately 0.5 μg or 1.5 μg of DNA. Spleens were removed approximately 3 weeks after the final inoculation and CTL activity was measured by chromium release assays. Target cells were transformed P815 mouse cells expressing Ebola virus GP or NP. Examples of results for individual mice are shown. Splenocytes from each mouse were also tested against untransformed control P815 cells and background lysis was less than 5% at *E*:*T* ratios for all mice (data not shown). (A) Mice were given two vaccinations with ~1.5 μg or 0.5 μg of GP DNA. (B) Mice were given three vaccinations with 0.5 μg or 3 μg of NP DNA or 3 μg of GP DNA. Specific lysis (percent lysis of test mice − percent lysis of control mice) is shown [65].

at 4-week intervals with a 20 μg DNA dose delivered by PMED. All vaccinated monkeys developed antibodies to Marburg virus as measured by ELISA. After challenge, all vaccinated and control monkeys had a febrile response, suggesting that the vaccine did not prevent infection. Nevertheless, vaccinated monkeys displayed normal liver enzyme profiles and only 2/6 vaccinated monkeys had measurable viremia. More importantly, 4/6 vaccinated monkeys survived challenge, while both control monkeys died. The results demonstrate that PMED DNA vaccine is immunogenic in monkeys, and provides partial protection against Marburg virus challenge.

Collectively, the findings suggest that PMED DNA vaccination may have application to filovirus vaccines. Vaccination with the GP gene of either Ebola or Marburg virus can elicit neutralizing antibodies, cellular immune responses, and protection against challenge. However, further optimization of formulation, dose and regimen may be warranted in order to achieve improved efficacy, more rapid onset of immunity and sufficient duration of immunity.

3.2. Flavivirus DNA vaccines

The family *Flaviviridae* represents small, spherical, enveloped, positive sense RNA viruses. The family includes yellow fever virus, tick-borne encephalitis viruses, dengue virus and Japanese encephalitis virus [130]. Flaviviruses are transmitted by arthropod vectors (ticks and mosquitos) and cause a number of serious diseases.

Several flaviviruses are associated with encephalitis syndromes, including Japanese encephalitis and tick-borne flaviviruses. Tick-borne encephalitis (TBE) is endemic in areas of Central and Eastern Europe, and is caused most commonly by the Russian Spring–Summer Encephalitis virus (RSSEV) and Central European Encephalitis virus (CEEV) strains of TBEV.

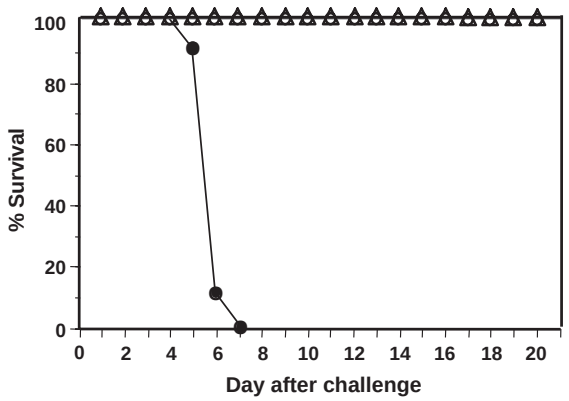

Fig. 4. Survival of mice vaccinated 2× with ~5 μg Ebola virus GP or NP DNA and challenged with Ebola virus. $n = 10$ for each group. Δ GP DNA; ○ NP DNA; ●control DNA.

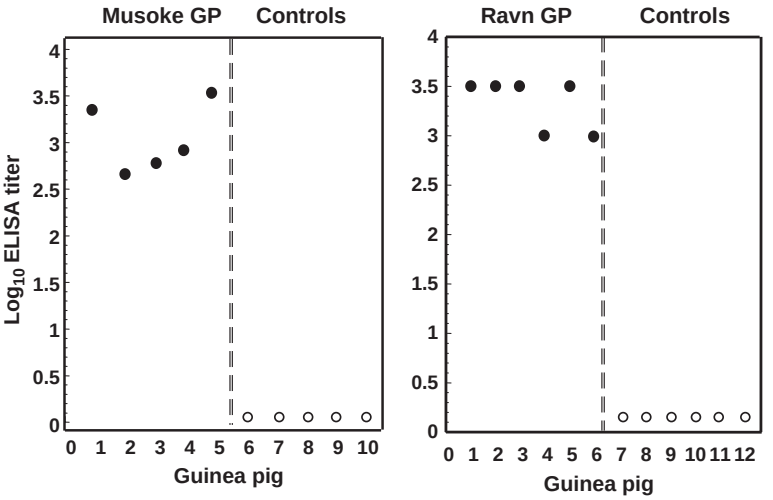

Fig. 5. ELISA antibody titers in guinea pigs vaccinated with Marburg virus PMED DNA vaccine. Strain 13 guinea pigs were vaccinated with GP DNA of Marburg virus strain Musoke. Hartley guinea pigs were vaccinated with GP DNA of Marburg virus strain Ravn. Guinea pigs were vaccinated at 4-week intervals three (Musoke) or four (Ravn) times by PMED administration of approximately 2.5 μg of DNA to each of four sites on the shaved abdomen (approximately 10 μg of DNA per vaccination). Serum was collected 3–4 weeks after the final vaccination, for quantitation of specific IgG antibody by ELISA, using cobalt-irradiated, purified Marburg virus, strains Musoke or Ravn, as antigen [31].

The incidence of TBE is approximately 11,000 new cases per year, primarily in Russia [131]. RSSEV and CEEV are serologically distinct, and are transmitted by different tick vectors. Infections are associated with outcomes including subclinical infection, biphasic febrile response, encephalitis and chronic disease (reviewed in [130,131]). Inactivated vaccines against tick-borne encephalitis are available in Europe but not the United States. TBE incidence in Europe has been significantly reduced by vaccination. The vaccines are associated with good safety, immunogenicity and efficacy, though reformulated versions of these vaccines are currently in development [132–134]. New TBE vaccines tested in animals include self-replicating non-infectious RNA [135], live-attenuated chimeric flaviviruses [136], and DNA vaccines (reviewed below). The PMED DNA vaccine approach may be particularly relevant to vaccines against pathogens such as TBE viruses, because the site of natural infection, via bite from infected tick, is localized to the skin.

Flaviviruses contain three structural proteins, a nucleocapsid, or core (C), a nonglycosylated membrane protein (M) and a glycosylated envelope protein (E) [130]. The M and E proteins form heterodimers, and the E protein contains the primary antigenic determinants. A DNA vaccine against TBE was constructed which encodes the genes for the M protein precursor (PrM) and the E protein [137]. This antigen combination was selected due to evidence that co-expressed PrM and E of CEEV can form immunogenic subviral particles that are protective in animals [138]. Protection by passive transfer of monoclonal antibodies directed against M and E has demonstrated a correlation between neutralizing antibodies and protection from tick-borne flaviviruses [138,139]. Mice received two immunizations of 0.5–1 μg RSSEV or CEEV DNA by PMED, delivered at 4-week intervals. All mice immunized with the DNA vaccines mounted neutralizing antibodies that were cross-reactive against both RSSEV and CEEV, and were protected from both RSSEV and CEEV challenge. One vaccination with 0.5 μg CEEV DNA provided protective immunity for at least 2 months and two or three vaccinations given at 4-week intervals vaccinations protected mice from CEEV challenge for at least 1 year after the initial vaccination. Following challenge, there was no increase in antibody titers and, in addition, no antibody was detected to the NS1 protein, which is present in the challenge virus, but not contained within the vaccine. The conclusion is that PMED

DNA vaccination prevented detectable replication of challenge virus in mice.

These same DNA vaccines were evaluated in cynomolgus macaques [140]. Monkeys were given three vaccinations by PMED at days 0, 30 and 70 with the individual DNA vaccines for RSSEV or CEEV or with a combination of the two vaccines. All of the vaccines elicited neutralizing antibodies in the monkeys. The antibody responses persisted for at least 15 weeks after the third vaccination and could be boosted with a fourth vaccination. Although monkeys are not a uniformly lethal model of TBEV, it was possible to evaluate protective efficacy of the vaccines by passive transfer of monkey sera to mice and challenge of the mice. Complete protection of mice to challenge with either RSSEV or CEEV was found to correlate with the neutralizing antibody titer of the monkey sera.

Other Flavirivuses such as dengue, yellow fever and West Nile viruses are not associated with encephalitis, but rather cause symptoms such as fever, arthralgia, rash and hemorrhagic fever [130]. Dengue virus is transmitted by the *Aedes* mosquito, and is endemic in tropical and subtropical areas (reviewed by [141]). The annual incidence of human dengue virus infection exceeds 100 million. Of these, the majority of infections are dengue fever, which is generally self-limiting and infrequently fatal. However, more than 250,000 cases are reported of dengue hemorrhagic fever, which is characterized by increased vascular permeability, leading to shock and death if not treated early and aggressively. Severe disease as a sequela of dengue virus infection occurs in about 2–6% of infected individuals.

Dengue virus vaccine development is complicated by the existence of four virus serotypes: DEN-1, DEN-2, DEN-3 and DEN-4. The serotypes are distinct, yet share limited serological cross-reactivity. Infection with dengue virus from one serotype results in lifelong protective immunity against the homologous serotype, but does not protect against heterologous serotypes. Furthermore, patients who are immune to one serotype have an elevated risk of developing dengue hemorrhagic fever when exposed to a heterologous serotype, a phenomenon termed antibody dependent enhancement (reviewed in [141]).

No animal model has been developed that reproduces the pathogenesis of dengue hemorrhagic fever. However, DNA vaccines encoding dengue virus antigens have been evaluated for immunogenicity and efficacy against challenge infection in rodents and nonhuman primates. DNA vaccines expressing the pre-M and truncated or full-length E gene and delivered i.d. are immunogenic in mice and provide partial protection from intracranial dengue challenge [142–144]. The vaccine containing full-length E is more immunogenic than truncated forms, likely because coexpression of both dengue antigens leads to production of highly immunogenic virus like particles in transfected cells. A DEN-1 pre-M/E DNA vaccine delivered i.d. was also found to be immunogenic and offer partial protection from dengue infection in rhesus macaques [145] and aotus monkeys [146]. Several approaches have been employed to enhance vaccine-induced neutralizing antibody responses and/or protective efficacy of parenteral dengue DNA vaccines, including incorporation of sequences to facilitate antigen trafficking [147,148], co-expression of GM-CSF [147] or GM-CSF and immunostimulatory sequences [149], DNA prime-protein boost strategies [150], or Biojector delivery [147]. Studies of i.m.-administered dengue DNA vaccines have demonstrated enhancement of neutralizing antibody responses by simultaneous administration of DNA and protein antigen [151]. Partial protection of mice from dengue challenge was also observed following i.m. immunization of a DNA vaccine expressing the dengue virus nonstructural protein NS1 [152].

Available data on dengue virus DNA vaccination by PMED are more limited. A DEN-2 DNA vaccine expressing pre-M and E elicited neutralizing antibodies and cytotoxic T lymphocyte responses in mice [67]. In a dose–response study in rhesus macaques, the PMED dengue vaccine elicited neutralizing antibodies in 3 of 3 monkeys receiving four 2 μg doses of DNA, 1 of 3 of animals receiving two 1 μg vaccine doses, and 0 of 3 monkeys receiving a single 1 μg vaccine dose. Despite the differences in antibody responses between the groups, all the monkeys receiving the two highest total doses were protected from viremia following challenge at 1 month after the final vaccination. In both of these groups, two monkeys had no viremia, and the third animal had one day of viremia, compared to 4.7 days of viremia in control monkeys. Additional monkeys immunized with two 1 μg vaccine doses were only partially

protected from viremia following challenge at 7 months post-vaccination.

These results demonstrate that a dengue DNA vaccine delivered by PMED can elicit protective immune responses, though the dosing regimen must be appropriately optimized. Further, such protective immune responses elicited by PMED DNA vaccination may not only include neutralizing antibodies, but also non-neutralizing antibodies and/or cell-mediated immune responses. Future studies will be required to investigate cross-protective efficacy, as well as vaccines incorporating all four dengue virus serotypes that will likely be required to avoid antibody-dependent enhancement of disease and provide an adequate level of efficacy against dengue hemorrhagic fever.

Protective efficacy has also been demonstrated with PMED DNA vaccines against the flaviviruses Murray valley encephalitis virus and yellow fever virus [153,154]. Murray valley prM/E vaccine elicited neutralizing antibodies, protection from challenge and extended duration of immunity [153]. A PMED DNA vaccine expressing the E glycoprotein of JEV was also protective against challenge [154]. Together, the preponderance of the data demonstrates protective efficacy of PMED DNA vaccines expressing either prM/E or E alone of diverse flaviviruses, which is mediated by the induction of neutralizing antibodies.

3.3. Poxvirus DNA vaccines

The history of smallpox vaccination is a story in successful disease eradication, using the live vaccinia virus for immunization. Recently renewed smallpox vaccination campaigns have raised concerns about severe side effects associated with the currently licensed live virus vaccine (Dryvax). Furthermore, the live virus vaccine can be inadvertently spread by autoinoculation (e.g., ocular exposure), or transmitted to other individuals [155]. Thus, there is interest in developing a new smallpox vaccine with an improved safety profile. Because the licensed vaccine is administered to the skin by scarification, there is a precedent for effective smallpox vaccination via the epidermal route. Delivery of smallpox DNA vaccines to the epidermis by PMED has the potential to elicit similar immune responses by targeting the anatomical site of natural smallpox infection.

The poxvirus double-stranded DNA genome is larger and much more complex than the genomes of the RNA viruses. Of the approximately 150–200 potential poxvirus genes, only a handful have been tested as potential subunit or DNA vaccine candidates in the vaccinia system. Identifying potential protective antigens of vaccinia virus is expected to extend to monkeypox and smallpox (variola) due to a high degree of homology among many of the open reading frames.

Four different antigens of vaccinia virus (L1R, A27L, A33R, and B5R) have been evaluated in DNA vaccination regimens. The antigens were selected to elicit immune responses against both of the major infectious forms of vaccinia virus, the intracellular mature virion (IMV), which is involved in host-to-host transmission of virus, and the extracellular enveloped virion (EEV), which is associated with virus dissemination within the host [156]. The intended goal was to target the infection at several levels: IMV introduced during primary infection and released from infected cells, and well as EEV circulating in the host and potentially virus-infected cells. Of the four antigens selected, two are present on IMV (L1R and A27L) and two are present on EEV (A33R and B5R). All four antigens are targets of protective antibodies [157–163].

While each individual antigen was able to elicit neutralizing antibodies, PMED DNA vaccination of mice with a combination of genes for IMV and EEV-associated antigens conferred greater protection than vaccination with any single gene [159,164,165]. Mice vaccinated by PMED with a DNA vaccine containing all four vaccinia virus genes were fully protected from vaccinia challenge. In addition, the 4-gene PMED DNA vaccine was immunogenic in rhesus macaques, and the antibody responses were cross-reactive with the orthologous monkeypox virus proteins [165].

These findings were recently extended to evaluation of efficacy against monkeypox challenge in nonhuman primates [166]. Four rhesus macaques were immunized by PMED with the DNA vaccine consisting of the four vaccinia genes A27L, A33R, L1R, and B5R. Monkeys received a 4-dose primary series, and a booster vaccination 1 to 2 years later. All three vaccinated monkeys survived lethal monkeypox challenge. Two additional monkeys receiving a DNA vaccine expressing only L1R developed severe disease

after challenge, but survived. In contrast, the three monkeys vaccinated with a negative control DNA vaccine developed grave monkeypox and died. These results suggest that a PMED DNA-based poxvirus vaccine encoding four antigens has promise as a safe and efficacious approach to smallpox prevention.

3.4. Alphavirus DNA vaccines

The alphaviruses are enveloped positive strand RNA viruses, and are a genus of the family Togaviridae (reviewed in [167]). Alphaviruses are transmitted by mosquito vectors and are associated with various disease syndromes, including acute arthritis (e.g., Sindbis virus, Ross River virus), encephalitis (e.g., eastern and western equine encephalitis), and systemic febrile illness (e.g., Venezuelan equine encephalitis, VEE). The majority of VEEV infections lead to clinical symptoms, primarily debilitating febrile illness, while encephalitis occurs in a small percent of infected subjects. Although no vaccines are licensed for human use, vaccines against Venezuelan, eastern and western equine encephalitis are available for veterinary use.

The alphaviruses encode two polyproteins, one of which is cleaved to produce the nonstructural proteins. The second polyprotein is translated from a subgenomic 26S mRNA which encodes the structural proteins, including capsid (C) and the envelope glycoproteins E1, E2 and E3. The majority of the neutralizing antibody response is directed against the E2 glycoprotein [168]. A candidate DNA vaccine expressing E2 glycoprotein of VEEV has been evaluated in mice [169]. Groups of mice were primed and boosted by either 0.6 or 4 μg of plasmid by PMED, or 50 μg of plasmid DNA with 0.25% bupivacaine hydrochloride by the i.m. or i.d. route. All mice from all groups seroconverted to the vaccine by day 21 post-prime. Mice were boosted on day 72. Antibody titers on days 93 through 176 were significantly higher in the group given 4 μg DNA by PMED than in the other groups. For all routes of administration, antibodies were primarily of the IgG1 isotype.

Protective immunity in mice has also been demonstrated using a PMED DNA vaccine representing the subgenomic (26S) mRNA of VEEV [31]. All vaccinated mice survived challenge by the SC route, while 80% of vaccinated mice survived aerosol challenge. Eighty percent of positive control mice vaccinated with the commercial VEEV vaccine (TC-83) survived both SC and aerosol challenges. Interestingly, protected TC-83-vaccinated mice had high levels of neutralizing antibody, while protected DNA-vaccinated mice had strong VEEV-specific antibodies, but low levels of neutralizing antibodies, suggesting that either non-neutralizing antibodies or cellular immune responses were mediating protection of DNA-vaccinated mice.

3.5. Hantavirus DNA vaccines

Hantaviruses are members of the family Bunyaviridae, genus Hantavirus (reviewed in [170]). Hantaviruses are associated with hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe, and with hantavirus pulmonary syndrome (HPS) in the United States and Central and South America. HFRS was first recognized in the West during the Korean War, when over 2400 United Nations soldiers contracted the diseases [171]. The viruses associated with HFRS include Seoul virus and Hantaan virus in Asia and Dobrava virus and Puumala virus in Europe, Scandinavia, and Western Russia. The incidence of HFRS is about 50,000–150,000 cases per year, primarily in Asia, with associated mortality rates of 0.1–10% dependent on the virus. Hantaan virus is associated with the highest case-fatality rate (5–15%), while Puumala virus infection leads to a milder disease termed nephropathia epidemica with a case-fatality rate of 0.1–0.3%. In 1993, HPS was recognized as a syndrome characterized by flu-like symptoms, but progressing to noncardiogenic pulmonary edema and shock. The incidence of HPS is much lower than HFRS, but the case-fatality rate of HPS is much higher at 40–60%. In addition, one of the HPS-causing hantaviruses found in South America, Andes virus, was shown to be transmitted from person-to-person [172].

Impetus for vaccine development in the West has been driven primarily by military needs, while in Asia, the focus has been on immunization of populations in endemic regions. Several inactivated virus vaccines have been licensed in Asia, while in the U.S., hantavirus vaccine research has focused on recombinant subunit, viral vectored, and DNA vaccine approaches (reviewed by [170]). The hantaviruses

contain three RNA genome segments: S, encoding the nucleoprotein, M, encoding the glycoproteins G1 and G2, and L, encoding the RNA-dependent RNA polymerase. Of these, neutralizing antibodies are directed against the G1 and G2 glycoproteins [173], and can provide passive protection against challenge infection [174]. A vaccinia-vectored Hantaan virus vaccine encoding both the M and S segments has been tested in Phases 1 and 2 clinical trials [175]. Neutralizing antibodies to Hantaan virus were detected in 72% of study subjects who did not have pre-existing antibodies to vaccinia virus. However, in subjects with pre-existing anti-vaccinia virus responses, only 26% mounted neutralizing antibody responses to Hantaan virus. These studies demonstrated the feasibility of achieving protective immunity to a hantavirus, provided that the issue of pre-existing immunity could be overcome.

DNA vaccines expressing hantaviral S or M segments have been tested. In an initial report, DNA vaccines expressing M or S sequences from Seoul virus (an HFRS-causing hantavirus carried by rats) were tested for immunogenicity in mice and for the ability to protect hamsters from infection [176]. In mice, more animals seroconverted and antibody titers were higher after immunization with a PMED DNA vaccine compared to i.m. DNA vaccination. Hamsters vaccinated with the DNA vaccine expressing Seoul virus M were protected from Seoul virus infection whereas those vaccinated with the DNA vaccine expressing Seoul virus S were not protected. These results were subsequently confirmed in another study comparing the DNA vaccines delivered by PMED to a vaccine comprised of Sindbis replicons expressing Seoul virus proteins [177]. Neutralizing antibody titers were elicited in all hamsters after a prime with the PMED DNA vaccine, while for the Sindbis replicon two doses were required for seroconversion. After three vaccine doses, antibody titers were highest for the DNA vaccine compared to the Sindbis vector, but all hamsters seroconverted to Seoul virus and all were protected from Seoul virus infection. The ability of the vaccine to cross-protect against Hantaan virus was also demonstrated. Neutralizing antibodies to Hantaan virus and protection from Hantaan virus infection was observed in 75% of Seoul virus M PMED DNA vaccinated hamsters, while less protection was observed with the Sindbis constructs.

A Hantaan virus M DNA vaccine has also been evaluated for immunogenicity in hamsters and rhesus macaques, and for homologous and heterologous protection against hantavirus infection in hamsters [178]. Vaccination of hamsters with the Hantaan virus M DNA vaccine using a 2 μg PMED DNA prime and 2 boosts prevented infection following challenge with Hantaan virus, as measured by lack of detectable immune response to the nucleocapsid protein of the challenge virus. Cross-neutralization and cross-protection activity was also demonstrated. Neutralizing antibodies elicited by either the Hantaan virus M DNA vaccine or the Seoul virus M DNA vaccine cross-neutralized Seoul virus, Hantaan virus and Dobrava virus, but not Puumala virus. In addition to homologous protection, the Seoul virus M DNA vaccine provided protection against Hantaan and Dobrava virus challenge, but not Puumala virus challenge. Likewise, the Hantaan virus M DNA vaccine protected against Seoul and Dobrava virus challenge. In rhesus macaques, both Seoul and Hantaan virus M vaccines elicited high neutralizing antibodies after 4 μg DNA as prime and 2 boosts, with titers persisting for 120 weeks following the final vaccination (Fig. 6). Titers and duration of immunity achieved by DNA vaccination compared favorably versus neutralizing titers in monkeys vaccinated with a recombinant vaccinia virus expressing Hantaan virus M and S. Upon a single re-immunization at 120 weeks with the DNA vaccine, a robust anamnestic response was observed (J. Hooper, personal communication).

DNA vaccines against hantaviruses associated with HPS have also been evaluated in preclinical models. A DNA vaccine expressing the M segment of Andes virus, a South American Hantavirus, was administered to rhesus macaques by PMED. After three vaccinations, the monkeys achieved very high levels of neutralizing antibodies to both Andes virus and Sin Nombre virus. Although monkeys become infected with Andes virus, they do not become noticeably ill [179]. Consequently, to determine if the antibodies elicited in the monkeys were protective, a series of passive-transfer experiments were performed in hamsters, using a recently described lethal HPS animal model [170]. Monkey sera injected into hamsters 1 day before challenge either provided sterile immunity or delayed the onset of

HPS and death. Importantly, sera injected on days 4 or 5 after challenge protected 100% of the hamsters from an otherwise lethal challenge dose of Andes virus [180]. These data not only provide evidence that a DNA-based PMED vaccine can offer protective immunity, but also offer hope for therapeutic intervention against HPS-causing hantaviruses with immune sera.

In other studies, a DNA vaccine encoding approximately 500 nucleotides of the M segment from Sin Nombre virus and a DNA vaccine encoding the full-length S segment were demonstrated to be immunogenic after PMED immunization of mice [181] and to protect deer mice, the natural host, against Sin Nombre infection [182]. Interestingly, no antibody response to the vaccine could be detected in protected mice.

Collectively, these results demonstrate robust and long-lived immunogenicity of DNA vaccines against diverse hantaviruses in nonhuman primates, the ability of the DNA vaccines to elicit high-titer neutralizing antibodies, and the cross-protective nature of these antibodies. The cross-neutralization and cross-challenge results illustrate the possibility of achieving sterilizing immunity against three of the four hantaviruses that cause HFRS using a monovalent vaccine.

3.6. Combination vaccines

One of the most promising applications of PMED DNA vaccine technology is to facilitate generation of multiagent DNA vaccines. Several studies have demonstrated the ability to co-deliver multiple antigens on the same plasmid by PMED DNA vaccination, including examples of dengue virus and tick-borne encephalitis virus vaccines described earlier [67,137]. Approaches have been described for constructing a vaccine comprised of four antigens derived from the same pathogen, cloned on separate plasmids, and co-delivered by PMED [165]. In the case of a vaccine comprised of four poxvirus antigens, immunogenicity was comparable when each plasmid was delivered as a separate immunizing dose, or when the four plasmids were each separately formulated, and then mixed and co-delivered in the same immunization. In this study, it was found that formulating the plasmids on separate gold particles was more effective than mixing the plasmids prior to coating on the gold, likely because the separate formulation increased the likelihood that different plasmids would be delivered to separate cells.

Feasibility of the application of a DNA vaccine strategy to multiagent combination vaccines was first

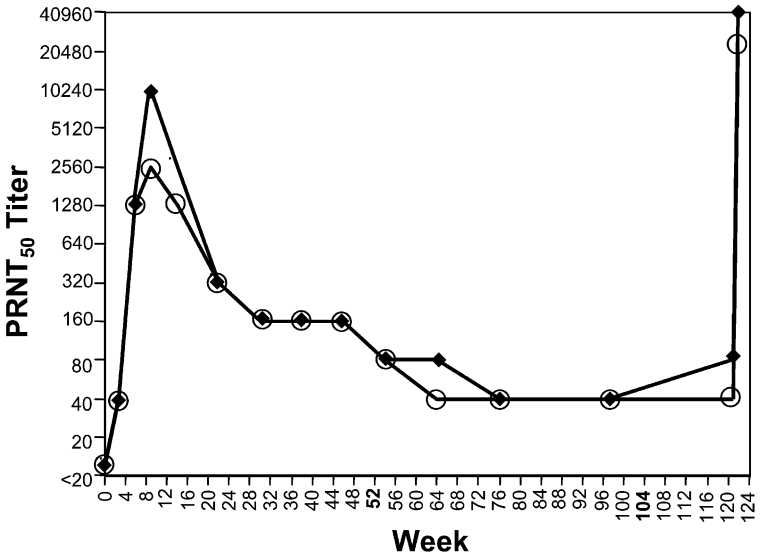

Fig. 6. Neutralizing antibodies to Hantaanvirus PMED DNA vaccine in monkeys. Two rhesus macaques were vaccinated by PMED on weeks 0, 3, and 6 with 4 μg of DNA expressing the M gene of Hantaan virus (pWRG/HTN-M(x)). Monkeys were boosted on week 120 with a single vaccination of the PMED DNA vaccine. The PRNT titer represents the reciprocal serum dilution that reduced virus plaque number by 50% [178].

shown for a combination DNA vaccine comprised of plasmids encoding antigens from influenza, herpes simplex and respiratory syncytial virus [183]. This study employed a DNA vaccination regimen that included PMED DNA immunization in combination with i.m. DNA immunization. Protection was demonstrated against each individual challenge and also against a multiagent challenge. The first description of protective efficacy mediated by a combination DNA vaccine encoding antigens from multiple pathogens and using PMED administration alone was reported for four plasmids separately encoding the protective antigen (PA) from *Bacillus anthracis*, the GP genes of Ebola virus or Marburg virus, and the 26S gene of VEEV, respectively [31]. Immunogenicity of each plasmid was first demonstrated individually in appropriate animal challenge modes. The monovalent anthrax PA PMED DNA vaccine elicited neutralizing antibodies and protected rabbits from lethal *B. anthracis* challenge. Guinea pigs vaccinated with the monovalent Marburg virus PMED DNA vaccine seroconverted and were protected from lethal Marburg virus infection. The monovalent Marburg DNA vaccine was also immunogenic in monkeys and provided partial protection from challenge. The monovalent Ebola virus DNA vaccine and the monovalent VEE vaccine were each shown to elicit protective immunity in mice.

To evaluate the feasibility of the combination vaccine, guinea pigs were vaccinated with either each individual DNA vaccine or with the tetravalent DNA vaccine. Each individual plasmid was delivered at a concentration of 5 μg, so guinea pigs receiving the tetravalent vaccine were vaccinated with 20 μg total DNA. Animals received a prime and two boosts at monthly intervals. Groups of guinea pigs were then challenged with either Ebola virus, Marburg virus or VEE virus. All guinea pigs in the respective monovalent and tetravalent vaccine groups seroconverted to Marburg and Ebola viruses (Table 2). Following challenge, the number of survivors was comparable between the monovalent and tetravalent DNA vaccines. It was speculated that the lower efficacy in this experiment compared to the previous study was due to a lower dose of vaccine used. For VEEV, two guinea pigs in the tetravalent group had very low antibody titers, however, all but one guinea pig were protected from challenge. Antibody titers were lower in the DNA vaccine groups compared to the commercial TC83 vaccine group, but survival was similar among the vaccines. Guinea pigs were not challenged with anthrax, however, antibody titers were comparable between the monovalent DNA vaccine group (2.5 $\log_{10}$ GMT) and the tetravalent DNA vaccine group (2.6 $\log_{10}$ GMT). Although optimization may be required, these initial results are promising in suggesting the feasibility of developing combination vaccines against dissimilar pathogens using PMED DNA vaccine technology.

Table 2
Comparison of single agent and multiagent DNA vaccines in guinea pigs[a]

Vaccine	Guinea pig strain	ELISA, Log_{10} GMT[b]	Viremic/Total	Survivors/Total	Mean day of death (range)
Ebola virus GP DNA	Strain 13	2.5	4/6	4/6	9 (8–9)
Multi DNA	Strain 13	2.7	2/5	3/5	16 (10–22)
Control DNA	Strain 13	< 1.6	6/6	0/6	11 (9–14)
Marburg virus GP DNA	Hartley	3.0	2/6	3/6	10 (8–11)
Multi DNA	Hartley	2.5	1/6	4/6	12 (10–13)
Control DNA	Hartley	< 1.5	6/6	0/6	12 (9–13)
VEEV 26S DNA	Hartley	2.7	1/6	6/6	NA
Multi DNA	Hartley	2.3	2/6	5/6	6 (NA)
Control DNA	Hartley	< 2.0	6/6	0/6	6 (5–7)
TC-83	Hartley	> 5.0	0/6	6/6	NA

NA—not applicable. VEEV—Venezuelan equine encephalitis virus.
[a] Adapted from reference [31].
[b] Geometric mean ELISA titer determined 3 weeks after the third vaccination.

4. Conclusions

DNA vaccines have been delivered by various routes, primarily i.m., i.d., or epidermal, via PMED. PMED has been shown to be a highly efficient mechanism of immunization, because it physically propels DNA directly into the cell cytoplasm or nucleus, and also targets a highly active immune inductive site, the epidermis of the skin. Due to the efficiency of intracellular delivery, 100- to 1000-fold less DNA is required to immunize with PMED compared to parenteral injection. Immune responses to PMED DNA vaccines are initiated by transfection of both professional and nonprofessional antigen-presenting cells in skin. Via direct transfection of antigen-presenting LCs, the antigen-encoding gene is transcribed, translated, and peptides are presented in the context of MHC class I complexes, to induce primary cellular immune responses and memory responses. When the DNA is deposited in keratinocytes, the antigen-encoding gene is transcribed, translated, and the product is usually secreted or inserted into the plasma membrane. Antigen produced in this manner can be processed by APCs via a cross-priming mechanism. Recent data have demonstrated that very few cells need to be transfected in order to elicit humoral, cellular and memory responses to the encoded antigen. These responses can be further enhanced by the addition of plasmids encoding immunostimulatory agents, including cytokines, chemokines, bacterial toxins, pro- or anti-apoptotic factors. Immune responses can also be augmented via the use of prime boost regimens or via the expression of antigens fused to specific intracellular targeting sequences. In several Phase 1 clinical trials, DNA vaccines for hepatitis B and malaria delivered by PMED elicited antibody and/or cellular immune responses, demonstrating clinical proof of concept of PMED DNA vaccination technology.

Potential application of PMED DNA vaccines to category A–C viral agents has been demonstrated in preclinical models. DNA vaccination elicits antibody responses to filoviruses, flaviviruses, alphaviruses, vaccinia virus, and hantaviruses, and cellular responses to the filoviruses Marburg and Ebola virus, and to the flavivirus dengue virus. Protective efficacy in rodent and/or nonhuman primate models of disease and infection has been demonstrated for PMED DNA vaccines expressing antigens from Ebola virus, Marburg virus, TBE virus, dengue virus, vaccinia virus, monkeypox virus, and hanta viruses responsible for both HFRS and HPS. In some cases (TBEV, hantavirus), the protection data are suggestive of sterilizing immunity. Robust duration of immunity to DNA vaccines has been demonstrated by the persistence of antibody responses for as long as 2 years following a PMED hantavirus vaccine, protection against challenge 1 year after vaccination with PMED DNA vaccine for TBEV, and partial protection at 9 months following vaccination with a PMED Ebola virus vaccine. Although, in many instances, it has been possible to correlate protection from experimental challenge with the level of neutralizing antibodies elicited by vaccination, in other cases, there is evidence that the protective immune mechanism differs between conventional vaccines and PMED DNA vaccines.

The concept of immunization against potential biological warfare agents using PMED DNA vaccines is now well established in the available preclinical models. Opportunities to enhance protective efficacy and duration of immunity of these vaccines remain to be explored. Clinical evaluation of vaccines for these agents can proceed using a platform that has been shown to be safe and immunogenic for DNA vaccines against other infectious diseases.

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